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(54) Title: USE OF A MELANOMA DIFFERENTIATION ASSOCIATED GENE (<i>mda-7</i>) FOR REVERSING A CANCEROUS PHENOTYPE (57) Abstract <p>This invention provides a method for reversing the cancerous phenotype of a cancer cell by introducing a nucleic acid having the melanoma differentiation associated gene (<i>mda-7</i>) into the cell under conditions that permit the expression of the gene so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the cancerous phenotype of a cancer cell by introducing the gene product of the above-described gene into the cancerous cell so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a pharmaceutical composition having an amount of a nucleic acid having the melanoma differentiation associated gene (<i>mda-7</i>) or the gene product of a melanoma differentiation associated gene (<i>mda-7</i>) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.</p>		

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**USE OF A MELANOMA DIFFERENTIATION ASSOCIATED GENE
(mda 7) FOR REVERSING A CANCEROUS PHENOTYPE**

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This application is a continuation-in-part of U.S. Serial No.08/696,573, filed June 16, 1996, the content of which is incorporated into this application by reference.

10 The invention disclosed herein was made with Government support under NCI/NIH Grant No. CA35675 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

15 Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

20 Full bibliographic citation for these references may be found at the end of each series of experiments.

Background of the Invention

25 Cancer is a complex multifactor and multistep process involving the coordinated expression and suppression of genes functioning as positive and negative regulators of oncogenesis (1-5). Direct cloning strategies, based on transfer of a dominant transforming or tumorigenic
30 phenotype, have identified positive acting oncogenes (6-9). In contrast, the detection and cloning of genes that suppress the cancer phenotype have proven more difficult and elusive (10-15). A direct approach for isolating genes directly involved in regulating growth
35 and differentiation involves subtraction hybridization between cDNA libraries constructed from actively growing cancer cells and cDNA libraries from cancer cells induced to lose proliferative capacity irreversibly and terminally differentiate (13,14). This experimental
40 strategy has been applied to human melanoma cells,

- 2 -

induced to terminally differentiate by treatment with recombinant human interferon β (IFN- β) and mezerein (MEZ), resulting in the cloning of novel melanoma differentiation-associated (*mda*) genes not previously described in DNA data bases (13,14). A direct role for specific *mda* genes in mediating growth and cell cycle control is apparent by the identification and cloning of *mda-6* (13-16), which is identical to the ubiquitous inhibitor of cyclin-dependent kinases p21 (17). The importance of p21 in growth control is well documented and this gene has been independently isolated, as WAF-1, CIP-1, and SDI-1, by a number of laboratories using different approaches (18-20). These studies indicate that specific genes associated with proliferative control are induced and may contribute to the processes of growth arrest and terminal differentiation in human cancer cells.

The *mda-7* gene was cloned from a differentiation inducer (IFN- β plus MEZ)-treated human melanoma (H0-1) subtracted library (13,14). The full-length *mda-7* cDNA is 1718 nucleotides, and the major open reading frame encodes a novel protein of 206 aa with an M_r of 23.8 kDa (21). Previous studies indicate that *mda-7* is induced as a function of growth arrest and induction of terminal differentiation in human melanoma cells (14,21). *mda-7* expression also inversely correlates with melanoma progression - i.e., actively growing normal human melanocytes express more *mda-7* than metastatic human melanoma cells (21). Moreover, *mda-7* is growth inhibitory toward human melanoma cells in transient transfection assays and in stable transformed cells containing a dexamethasone (DEX)-inducible *mda-7* gene (21). These studies indicate that *mda-7* may contribute to the physiology of human melanocytes and melanomas, and this gene has growth suppressive properties when overexpressed in human melanoma cells.

- 3 -

The *mda-7* gene was also described in the International Patent Cooperation Treaty Application No. PCT/US94/12160, international filing date, October 24, 1994 with International Publication No. WO95/11986, the content of which is incorporated into this application by reference.

This invention reports that *mda-7* is a potent growth suppressing gene in cancer cells of diverse origin, including breast, central nervous system, cervix, colon, prostate and connective tissue. An inhibition in colony formation occurs in cancer cells containing defects in their p53 and/or retinoblastoma (RB) genes or lacking p53 and RB expression. In contrast, expression of *mda-7* in normal human mammary epithelial cells, human skin fibroblasts and rat embryo fibroblasts induces quantitatively less growth suppression than in cancer cells. When stably expressed in human cervical carcinoma (HeLa) and prostate carcinoma (DU-145) cells, *mda-7* has a negative effect on growth and transformation-related properties. The effects of *mda-7* on HeLa cells are reversible following abrogation of the MDA-7 protein by infection with a genetically modified Ad5 vector expressing an antisense *mda-7* gene. These observations indicate that *mda-7* is a novel growth suppressing gene with a wide range of inhibitory actions in human cancers manifesting different genetic defects.

Summary of the Invention

5 This invention provides a method for reversing the cancerous phenotype of a cancer cell by introducing a nucleic acid including a melanoma differentiation associated gene (*mda-7*) into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the
10 cancerous phenotype of cancer cell in a subject by introducing the above-described nucleic acid into the subject's cancerous cell.

15 This invention also provides a method for reversing the cancerous phenotype of a cancer cell by introducing the gene product of a melanoma differentiation associated gene (*mda-7*) into the cancer cell so as to thereby reverse the cancerous phenotype of the cell. This invention also provides a method for reversing the
20 cancerous phenotype of a cancer cell in a subject by introducing the above-described gene product into the subject's cancerous cell.

25 This invention also provides a pharmaceutical composition having an amount of a nucleic acid including a melanoma differentiation associated gene (*mda-7*) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition having an amount of
30 the gene product of the above-described gene effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

Brief Description of the Figures

5 **Figure 1.** Effect of *mda-7* expression on hygromycin resistant colony formation in HeLa cells. HeLa cells were transfected with 10 μ g of pREP4 vector (RSV-vector), *mda-7* cloned in an antisense orientation in the pREP4 vector (RSV-MDA-7-Antisense), or *mda-7* cloned in a sense orientation in the pREP4 vector (RSV-MDA-7-Sense) and selected in media containing 100 μ g of hygromycin.

15 **Figure 2.** Effect of antisense *mda-7* on monolayer growth of pREP4 vector HeLa cl 1 and *mda-7* (S) expressing HeLa cl 2 cells. HeLa cl 1 (pREP4 vector transformed HeLa clone) and HeLa cl 2 (*mda-7* expressing HeLa clone) cells were grown in the absence or following infection with 10 plaque forming units/cell with a recombinant type 5 adenovirus (Ad5) expressing antisense *mda-7* [Ad.*mda-7* (AS)]. Results are the average cell number from triplicate samples that varied by $\leq 10\%$.

25 **Figure 3.** Effect of antisense *mda-7* on the high molecular weight-MDA-7 complexing (HMC) protein, the MDA-7 protein and the actin protein in HeLa, HeLa cl 1, and HeLa cl 2 cells. HeLa and HeLa cl 1 (pREP4 vector transformed HeLa clone) were uninfected (-) or infected (+) with 10 plaque forming units/cell of Ad.*mda-7* (AS) for 96 hr labeled with [35 S] methionine, and the levels of the HMC, MDA-7 and actin proteins were determined by immunoprecipitation analysis. For HeLa cl 2 (*mda-7* expressing HeLa clone), the effect of infection with 10 plaque forming units/ml of Ad.*mda-7* (AS) on protein levels was determined by immunoprecipitation analysis of

- 6 -

[³⁵S] methionine labeled cell lysates after +24, +48, +72 and +96 hr. The effect of infection of HeLa cl 2 cells with the control mutant Ad5, H5dl 434, was determined by immunoprecipitation analysis of [³⁵S]methionine labeled cell lysates 96 hr after infection with 10 plaque forming units/cell.

5

Figures 4A & 4B Synthesis of *mda-7* RNA and protein in DU-145 clones containing a DEX-inducible *mda-7* gene.

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Figure 4A. Cells were grown in the absence or presence of 10⁻⁶ M DEX for 96 hr, and total RNA was isolated, subjected to Northern blotting and probed with *mda-7*, a neomycin resistance (Neo^R) gene and GAPDH.

15

Figure 4B. Cells were grown in the absence or presence of 10⁻⁶ M DEX for 96 hr, cellular proteins were labeled with [³⁵S] methionine and immunoprecipitated with antibodies recognizing MDA-7 and actin proteins.

20

Figure 5 Inhibition of growth of established human cervical cancer (HeLa) xenografts in athymic nude mice.

25

Figure 6 Effect of Ad.*mda-7* S on HeLa tumor volume ratios. The result indicates that Ad.*mda-7* S can inhibit tumor progression in vivo in nude mice.

30

Detailed Description of the Invention

In order to facilitate an understanding of the Experimental Details section which follows, certain
5 frequently occurring methods and/or terms are described in Sambrook, et al. (45).

This invention provides a method for reversing the cancerous phenotype of a cancer cell which comprises
10 introducing a nucleic acid comprising a melanoma differentiation associated gene (*mda-7*) into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell.

15 This invention also provides a method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing a nucleic acid molecule comprising a melanoma differentiation associated gene (*mda-7*) into
20 the subject's cancerous cell under conditions permitting expression of the gene in the subject's cells so as to thereby reverse the cancerous phenotype of the cell.

25 Methods to introduce a nucleic acid molecule into cells have been well known in the art. Naked nucleic acid molecule may be introduced into the cell by direct transformation. Alternatively, the nucleic acid molecule may be embedded in liposomes. Accordingly, this invention provides the above methods wherein the nucleic
30 acid is introduced into the cells by naked DNA technology, adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retroviral vectors, vaccinia virus vector, liposomes, antibody-coated liposomes, mechanical
35 or electrical means. The above recited methods are merely served as examples for feasible means of introduction of the nucleic acid into cells. Other

methods known may be also be used in this invention.

5 In an embodiment of the above methods, the melanoma differentiation associated gene (*mda-7*) is linked to a regulatory element such that its expression is under the control of the regulatory element. In a still further embodiment, the regulatory element is inducible or constitutive. Inducible regulatory element like an inducible promoter is known in the art. Regulatory
10 element such as promoter which can direct constitutive expression is also known in the art.

In a separate embodiment, the regulatory element is a tissue specific regulatory element. The expression of
15 the *mda-7* gene will then be tissue-specific.

In another embodiment of the above-described methods, the cancer cell is characterized by the presence within the cancer cell of a defective tumor suppressor gene. The
20 defective tumor suppressor gene includes, but is not limited to, a p53, a retinoblastoma (RB) or a p16^{ink4a} gene.

In an embodiment of the above-described methods, the cancer cell is characterized by the presence within the
25 cancer cell of a dominant acting oncogene. Specifically, the dominant acting oncogene may be a Ha-ras, mutant p53 or human papilloma virus genes. The Ha-ras is a Harvey virus ras oncogene.

30 In an embodiment of the above methods, the nucleic acid comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector and vaccinia
35 virus vector. In a preferred embodiment, the adenovirus vector is a replication-defective adenovirus vector expressing *mda-7*, designated Ad.*mda-7* S. In another

embodiment, the adenovirus vector is a replication-competent adenovirus vector.

5 This invention also provides a method for reversing the cancerous phenotype of a cancer cell which comprises introducing the gene product of a melanoma differentiation associated gene (*mda-7*) into the cancerous cell so as to thereby reverse the cancerous phenotype of the cell.

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This invention further provides a method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing the gene product of a melanoma differentiation associated gene (*mda-7*) into the
15 subject's cancerous cell so as to thereby reverse the cancerous phenotype of the cell.

In an embodiment of the above-described methods, the cancer cell includes, but is not limited to, a breast,
20 cervical, colon, prostate, nasopharyngeal, lung connective tissue or nervous system cell. The cancer cell further includes cells from glioblastoma multiforme, lymphomas and leukemia.

25 This invention also provides a pharmaceutical composition which comprises an amount of a nucleic acid comprising a melanoma differentiation associated gene (*mda-7*) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

30

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers. The pharmaceutical composition may be constituted into any form suitable for the mode of
35 administration selected. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid

- 10 -

forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

5 In an embodiment, the nucleic acid comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV virus, retrovirus vector and vaccinia virus vector. In a
10 preferred embodiment, the adenovirus vector is a replication-defective adenovirus vector expressing *mda-7*, designated Ad.*mda-7* S. In another embodiment, the adenovirus is a replication-competent adenovirus vector.

15 This invention also provides a pharmaceutical composition comprising an amount of the gene product of a melanoma differentiation associated gene (*mda-7*) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.

20 In an embodiment of the above-described methods, the cancer cell includes, but is not limited to, a breast, cervical, colon, prostate, nasopharyngeal, lung connective tissue and nervous system cells. The cancer
25 cell further includes cells from glioblastoma multiforme, lymphomas and leukemia.

This invention will be better understood from the Experimental Details which follow. However, one skilled
30 in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Cancer is a disease characterized by defects in growth control, and tumor cells often display abnormal patterns of cellular differentiation. The combination of recombinant human fibroblast interferon and the antileukemic agent mezerein corrects these abnormalities in cultured human melanoma cells resulting in irreversible growth arrest and terminal differentiation. Subtraction hybridization identifies a melanoma differentiation associated gene (*mda-7*) with elevated expression in growth arrested and terminally differentiated human melanoma cells. Colony formation decreases when *mda-7* is transfected into human tumor cells of diverse origin and with multiple genetic defects. In contrast, the effects of *mda-7* on growth and colony formation in transient transfection assays with normal cells, including human mammary epithelial, human skin fibroblast and rat embryo fibroblast, is quantitatively less than that found with cancer cells. Tumor cells expressing elevated *mda-7* display suppression in monolayer growth and anchorage independence. Infection with a recombinant type 5 adenovirus expressing antisense *mda-7* eliminates *mda-7* suppression of the *in vitro* growth and transformed phenotype. The ability of *mda-7* to suppress growth in cancer cells not expressing or containing defects in both the retinoblastoma (RB) and p53 genes indicates a lack of involvement of these critical tumor suppressor elements in mediating *mda-7*-induced growth inhibition. The lack of protein homology of *mda-7* with previously described growth suppressing genes and the differential effect of this gene on normal versus cancer cells suggests that *mda-7* may represent a new class of cancer growth suppressing genes with antitumor activity.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human carcinoma cell lines, including MCF-7 and T47D (breast), LS174T and SW480 (colorectal), HeLa (cervical), DU-145 (prostate), and HONE-1 (nasopharyngeal) (9,22-25), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10) at 37°C in a 5% CO₂/95% air-humidified incubator. Additional human cell types including HBL-100 (normal mammary epithelial), H0-1 and C8161 (melanoma), GBM-18 and T98G (glioblastoma multiforme) and Saos-2 (human osteosarcoma) were maintained under similar conditions. Early passage normal human mammary epithelial cells (HMEC; passages 10-12) were obtained from Clonetics Corporation (San Diego, CA). HMEC cells were maintained in serum-free medium as described by Clonetics Corporation. CREF-Trans 6 (cloned Fischer rat embryo fibroblast) (9,26) and CREF Ha-ras (CREF cells transformed by the Ha-ras (T24) oncogene) (27) were cultured in DMEM-5. HeLa cl 1 is a hygromycin resistant (Hyg^R) Rous Sacroma virus RSV vector (pREP4) (Invitrogen) transformed HeLa clone. HeLa cl 2 is a Hyg^R *mda-7* expressing HeLa clone. HeLa cl 1 and HeLa cl 2 cells were constructed as described (12,21) and maintained in DMEM-10 containing 100 µg/ml of hygromycin. DU-145 cl 6 and DU-145 cl 7 cells contain a DEX-inducible *mda-7* gene (cloned in a pMAMneo vector) (Clontech) (21) and are maintained in DMEM-10 containing 200 µg/ml G418.

Subtraction Hybridization, Plasmids, Expression Vector Constructs, and Northern Hybridization. Identification and cloning of *mda-7* by subtraction hybridization was achieved as described (13). A full-length *mda-7* cDNA was isolated by screening a recombinant IFN-β plus MEZ-treated H0-1 cDNA library (13) and using the procedure of rapid amplification of cDNA ends as described (15). An *mda-7* cDNA fragment (nucleotide position 176-960) containing the open reading frame was

- 13 -

amplified with PCR and cloned into pCRII™ (Invitrogen) by TA cloning. The orientation of the inserts in the vectors was determined by restriction mapping. The human cell expression constructs were made by cloning *Kpn I* - *Xho I* fragments from the PCR™ vectors into pREP4 vector (Invitrogen) downstream of a RSV promoter in a sense [mda-7 (S)] or antisense [mda-7 (AS)] orientation. Alternatively, the *mda-7* gene fragment was cloned into the pMAMneo (Clontech) vector in a sense and antisense orientation. RNA isolation and Northern blotting were performed as described (9,12,13,21).

Monolayer Growth, Anchorage-Independence and DNA-Transfection Assays. Monolayer and anchorage-independent growth assays were performed as previously described (8,12,26). To study the effect of *mda-7* on monolayer colony formation the vector [pREP4 (RSV)] containing no insert, *mda-7* (S) or *mda-7* (AS) expression constructs were transfected into the various cell types by the lipofectin method (GIBCO/BRL) and hygromycin resistant colony formation or cell growth in hygromycin was determined (12,21).

Construction of Antisense-*mda-7* Adenovirus Vector. The recombinant replication-defective Ad.*mda-7* (AS) was created in two steps. First, the coding sequence of the *mda-7* gene was cloned into a modified Ad expression vector pAd.CMV (28). This contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus (CMV) immediate early promoter, DNA encoding splice donor and acceptor sites, cloning sites for the desired gene (in this case *mda-7*), DNA encoding a polyA signal sequence from the beta globin gene, and approximately 3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the CMV immediate early gene promoter, and appropriate RNA

processing (28). The recombinant virus was created in vivo in 293 cells (29) by homologous recombination between *mda-7*-containing vector and plasmid JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322 (30). JM17 gives rise to Ad genomes in vivo but they are too large to package. This constraint is relieved by recombination with the vector to create a packageable genome (30), containing the gene of choice. The recombinant virus is replication defective in human cells except 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures (31).

15

Peptide Antibody Production and Immunoprecipitation Analyses. Peptide antibodies were prepared against PSQENEMFSIRD as described (21). Logarithmically growing HeLa, HeLa cl 1 (Hyg^R pREP4 vector control HeLa clone), and HeLa cl 2 [pREP4-*mda-7* (S) transfected Hyg^R *mda-7* expressing HeLa clone] cells were either untreated or infected with 10 plaque forming units of control adenovirus (H5dl434) (32) or a recombinant adenovirus expressing *mda-7* (AS) [Ad.*mda-7* (AS)]. At various times after infection, cultures were starved of methionine for 1 hr at 37°C in methionine-free medium, cells were concentrated by pelleting and labeled for 4 hr at 37°C in 1 ml of the same medium with 100 µCi (1Ci=37GBq) of ³⁵S (NEN; Express ³⁵S). Immunoprecipitation analyses with 2 µg of MDA-7 peptide rabbit polyclonal antibody or actin monoclonal antibody (Oncogene Sciences) were performed as described (15,21).

30

EXPERIMENTAL RESULTS

35

Enhanced Growth Inhibitory Properties of *mda-7* in Human Cancer Cells and Ha-ras-Transformed Rat Embryo Fibroblast Cells. DNA transfection assays were performed to evaluate

- 15 -

the effect of elevated expression of *mda-7* on cell growth. When transfected into human cervical carcinoma (HeLa) cells, the *mda-7* (S) construct results in a 10- to 15-fold reduction in Hyg^R colonies in comparison with the pREP4 vector and *mda-7* (AS) construct transfected cultures (Fig. 1 and Table 1).

Table 1 Effect of *mda-7* on monolayer colony formation of human cancer, normal rat embryo fibroblast (CREF) and Ha-ras-transformed CREF cells.

Cell Type	RSV-Vector ^a	RSV- <i>mda-7</i> (S) ^b	RSV- <i>mda-7</i> (AS)
Human cancer cell lines ^c			
MCF-7 (Breast- Ca)	118 ± 24	42 ± 16 (3.5)	146 ± 20
T47D (Breast- Ca)	172 ± 9	44 ± 7 (4.2)	186 ± 28
HeLa (Cervix- Ca)	1571 ± 446	117 ± 107 (15.2)	1771 ± 385
LS174T (Colorectal-Ca)	130 ± 14	30 ± 3 (5.4)	160 ± 15
HONE-1 (Nasopharyngeal-Ca)	219 ± 19	71 ± 8 (3.5)	250 ± 19
DU-145 (Prostate-Ca)	174 ± 18	54 ± 8 (3.1)	166 ± 12
T98G (Glioblastoma)	99 ± 9	32 ± 4 (3.6)	115 ± 14
Saos-2 (Osteosarcoma)	126 ± 22	35 ± 6 (3.9)	138 ± 14
Rat embryo fibroblast			
CREF (normal rat embryo)	60 ± 10	35 ± 5 (1.7)	66 ± 7
CREF-ras (transformed)	147 ± 16	25 ± 4 (6.0)	151 ± 16

^a Logarithmically growing cells were seeded at 1 X 10⁶ per 100-mm plate and transfected with 10 µg of

- 16 -

- vector [pREP4 (RSV)] containing no insert, *mda-7* (S), or *mda-7* (AS). After 24 hr, cells were replated at approximately 2×10^5 cells per 100-mm plate in medium containing 100 $\mu\text{g/ml}$ of hygromycin. Medium was changed every 3 or 4 days and plates were fixed in formaldehyde and stained with Giemsa at day 14 or 21. Colonies containing 50 or more cells were enumerated. Values shown are the average Hyg^R colonies formed in four to five replicate plates \pm S.D.
- ^b Values in parentheses indicate fold-decrease in colony formation versus RSV-*mda-7* (AS) transfected cells.
- ^c MCF-7, T47D, HeLa, LS174T, DU-145 and HONE-1 are human carcinoma (Ca) cell lines isolated from the indicated anatomical site. T98G is a human glioblastoma multiforme cell line. CREF-ras is a Ha-ras (T24) oncogene transformed CREF clone.
- In addition to forming fewer colonies, *mda-7* (S) colonies are generally smaller in size than corresponding Hyg^R colonies resulting after transfection with the pREP4 vector or *mda-7* (AS) constructs (Fig. 1). When transfected into additional human cancer cell lines *mda-7* (S) constructs reduce Hyg^R colony formation by 3- to 10-fold (Table 1). These include human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (H0-1 and C8161), glioblastoma multiforme (GBM-18 and T98G) and osteosarcoma (Saos-2). As observed with HeLa cells, the average sizes of Hyg^R colonies that form after transfection with *mda-7* (S) constructs are smaller than those formed following transfection with the empty pREP4 vector or *mda-7* (AS) constructs. These results demonstrate that *mda-7* is a potent growth suppressing gene when over-expressed in a wide spectrum of histologically distinct human cancers.

- 17 -

To determine if *mda-7* also inhibits the growth of normal cells and whether this effect is quantitatively similar to that observed with human cancer cells, transient DNA transfection assays were performed with passage 10 to 12 normal human mammary epithelial (HMEC) cells, the normal breast epithelial cell line HBL-100, normal human skin fibroblasts (passage 21) and a cloned normal rat embryo fibroblast cell line (CREF-Trans 6) (7,8). Since HMEC, HBL-100 and normal human skin fibroblasts do not form well-defined colonies at high frequencies, even when using a feeder-layer, the effect on total cell number after transfection with the different RSV constructs and growth for two and three weeks in hygromycin was determined. Using this approach, an approximate 1.1 to 1.6-fold decrease in HMEC, an approximate 1.1 to 1.2-fold decrease in HBL-100 and an approximate 1.3 to 2.1-fold decrease in normal human skin fibroblast cell number was observed (three independent experiments with each cell type) in *mda-7* (S) versus *mda-7* (AS) or pREP4 vector transfected normal cells, respectively. In contrast, using a similar experimental protocol with T47D human breast carcinoma cells, growth was inhibited following transfection with the *mda-7* (S) construct approximately 3.2 to 5.2-fold in comparison with vector- and antisense-transfected cells. In the case of CREF-Trans 6 cells, the difference in Hyg^R colony formation for six independent transfection assays between *mda-7* (S) versus *mda-7* (AS) and vector transfected cells ranged from 0.5 to 2.8-fold (Table 1). In contrast, transfection of *mda-7* (S) constructs into Ha-ras transformed CREF cells reduced colony formation by ~6 to 8-fold (Table 1). These results indicate that *mda-7* is quantitatively less effective in reducing growth and colony formation in normal human and normal rodent cells than in human cancer and Ha-ras-transformed rat embryo cells.

Effect of Stable and Inducible *mda-7* Expression and

Antisense Inhibition of *mda-7* Expression on Cell Growth and the Transformed Phenotype. To determine the reason for low frequency HeLa cell survival after transfection with the *mda-7* (S) gene, ten independent Hyg^R colonies were isolated following transfection with the *mda-7* (S) construct. Of the 10 clones analyzed by Northern blotting for *mda-7* expression, 7 clones did not express detectable *mda-7* mRNA, 2 clones expressed low levels of *mda-7* mRNA and 1 clone (designated HeLa cl 2) displayed high levels of *mda-7* mRNA. In contrast, all of the clones displayed comparable levels of Hyg^R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. When compared with parental HeLa cells or an pREP4 vector HeLa clone (designated HeLa cl 1), HeLa cl 2 (*mda-7* expressing) cells grew at a reduced rate (Fig. 2). When grown in agar, uncloned HeLa and HeLa cl 1 cells grew with approximately 42% efficiency, whereas HeLa cl 2 (*mda-7* expressing) cells grew with approximately 25% efficiency and the average sizes of colonies were smaller than observed with parental HeLa and pREP4 vector HeLa cl 1 cells. These results indicate that HeLa survival after transfection with *mda-7* results primarily from the lack of or low levels of *mda-7* expression. However, in HeLa cells that stably express elevated *mda-7*, growth in monolayer culture and anchorage-independence are reduced.

To determine if the reduction in *in vitro* growth and transformation suppression found in HeLa cl 2 (*mda-7* expressing) are a direct consequence of *mda-7* expression, an antisense strategy was used to directly inhibit *mda-7* expression. A recombinant Ad5 vector containing the *mda-7* gene cloned in an antisense orientation [Ad.*mda-7* (AS)] was constructed. Infection of HeLa cl 2 (*mda-7* expressing), but not HeLa cl 1 (pREP4 vector, non-*mda-7* expressing) or parental HeLa, with Ad.*mda-7* (AS) increases growth rate and agar cloning efficiency (from approximately 25 to approximately 44%) (Fig. 2). In

- 19 -

contrast, the control mutant Ad5 vector (H5dl434), not containing the *mda-7* gene, does not affect monolayer or agar growth of parental HeLa, HeLa cl 1 or HeLa cl 2 cells (data not shown).

5 Using *mda-7*-specific peptide antibodies produced in rabbits and immunoprecipitation analyses, the HeLa cl 2 (*mda-7* expressing) cells contain elevated levels of the MDA-7 approximately 24 kDa protein and a high molecular
10 weight complexing (HMC) protein of approximately 90 to 110 kDa (Fig. 3). Infection with Ad.*mda-7* (AS), but not the H5dl434 control non-*mda-7* expressing virus, results in a temporal decrease in both the ~24 kDa MDA-7 protein and the HMC protein (21) (Fig. 3). Reduced levels of both
15 proteins are seen by 48 hr and remain suppressed over a 96 hr period after infection with Ad.*mda-7* (AS). In contrast, actin levels remain unaltered following viral infection. These findings indicate that antisense inhibition of MDA-7 protein expression in HeLa cl 2
20 (*mda-7* expressing) can directly extinguish *mda-7* induced growth suppression and inhibition in anchorage-independent growth.

To confirm the suppressive effect of *mda-7* on cell
25 growth, DU-145 human prostate cancer cells were engineered to express a DEX-inducible *mda-7* gene. When DU-145 cl 6 or cl 7 cells [containing a DEX-inducible *mda-7* (S) gene], but not parental DU-145 cells, are grown for 24 to 96 hr in the presence of 10^{-6} M DEX, *mda-7* mRNA
30 and protein (including the HMC protein) are induced (Fig. 4). In contrast, DEX does not alter neomycin resistance (Neo^R) gene expression in DU-145 cl 6 and cl 7 cells or GAPDH expression in any of the cells tested (Fig. 4). Induction of *mda-7* expression in DU-145 cl 6 and cl 7
35 cells by growth in 10^{-6} M DEX results in approximately 50% reduction in cell number after 96 hr versus growth in the absence of DEX. In contrast, no significant growth

- 20 -

inhibition occurs when parental DU-145 or pMAMneo vector transformed DU-145 cells are grown for 96 hr in medium containing 10^{-6} M DEX (data not shown). These data indicate that ectopic expression of *mda-7* can directly alter cell growth in prostate cancer cells.

EXPERIMENTAL DISCUSSION

Subtraction hybridization identified *mda* genes with elevated expression in growth arrested and terminally differentiated human melanoma cells (13,14,21). Determining the function of these *mda* genes will be paramount in defining the molecular basis of growth control and terminal differentiation in human melanoma and other cell types. The *mda-7* gene (14,21) is now shown to be a ubiquitous growth suppressing gene when transiently or stably expressed in a wide array of human cancer cell lines. This finding extends previous observations indicating growth inhibitory properties of the MDA-7 protein in human melanoma cells (21). In contrast to its effects on cancer cells, transfection of *mda-7* into normal human mammary epithelial, normal human skin fibroblast and normal rat embryo fibroblast cells results in quantitatively less growth suppression. Like another *mda* gene, *mda-6* (p21), *mda-7* expression is also inversely correlated with melanoma progression, with elevated levels of both *mda-6* (p21) and *mda-7* present in normal human melanocytes relative to metastatic human melanoma cells (14-16,21). Since normal melanocytes still retain proliferative capacity, although at a reduced rate relative to melanoma cells, it is possible that both *mda-6* (p21) and *mda-7* function as negative regulators of the progression phenotype in melanocyte/melanoma lineage cells (14-16,21). Moreover, the elevated expression of both *mda-6* (p21) and *mda-7* in terminally differentiated and irreversibly growth arrested human melanoma cells, suggests that these genes may also be important regulators of the terminal differentiation phenotype

(13-16,21).

The mechanism by which *mda-7* elicits its growth suppressive effects on human cancer cells is not presently known. The structure of *mda-7* does not provide insight into potential function, since no sequence motifs are present that would suggest a potential mode of action. The effect of *mda-7* on cell growth can be distinguished from the extensively studied tumor suppressor gene p53 (33,34). Transient expression of p53 in the mutant p53 containing T47D human breast carcinoma cell line results in growth suppression, whereas transfection of a wild-type p53 gene into the wild-type p53 containing MCF-7 human breast carcinoma cell line does not induce growth inhibition (34). In contrast, *mda-7* induces similar growth suppression in both T47D and MCF-7 cells (Table 1). Growth inhibition by *mda-7* can also be disassociated from that observed with the retinoblastoma gene (pRB), the pRB-associated p107 gene and the putative tumor suppressor gene p16^{ink4} (25,35). Overexpression of pRB and p107 inhibit cellular proliferation in specific cell types and in a cell cycle-dependent manner (35-37). Transfection of pRB or p107 into the human glioblastoma cell line T98G that contains an apparently normal RB gene (25) does not induce growth suppression (35,37), whereas transient *mda-7* (S) expression reduces T98G colony formation (Table 1). At the present time, the growth inhibitory effect of *mda-7* cannot be distinguished from growth suppression induced by the RB family member p130/pRB2, which also inhibits proliferation in T98G cells (25). The p16^{ink4} gene induces growth arrest in cells containing a functional RB gene (35,37), whereas *mda-7* growth suppression occurs in cells containing normal, abnormal or non-functional RB genes. Transfection of *mda-7* into the DU-145 human prostate carcinoma cell line that contains a mutated RB gene (38) and Saos-2 human osteosarcoma cells that do not

express RB (or wild-type p53) results in an inhibition in colony formation (Table 1). Similarly, induction of *mda-7* expression in stable DEX-inducible *mda-7* transformed DU-145 clones results in growth suppression. These findings indicate a lack of dependence on a functional RB gene for growth inhibition by *mda-7*. Taken together these studies demonstrate that the inhibitory effect of *mda-7* occurs by a mechanism that is distinct from the mode of action of the two most extensively studied tumor suppressor genes, p53 and pRb, and the putative tumor suppressor gene p16^{ink4}.

Several genes have been identified that display elevated expression as a function of growth arrest or DNA damage in mammalian cells (39,40). Three growth arrest and DNA damage inducible (*gadd*) genes, *gadd45*, *gadd153* and *gadd34*, the closely related myeloid differentiation primary response (MyD118) gene (41) and the wild-type p53 inhibiting gene mdm-2 (42) are upregulated in cells by treatment with the DNA damaging agent methyl methanesulfonate (MMS) (40). The *gadd45* and growth arrest-specific gene (*gas1*) (43,44) are induced by maintaining cells at confluence, serum-starving cells or growing cells in low serum (40,43,44). In contrast, *mda-7* mRNA expression is not induced in human melanoma cells following treatment with methyl methane sulfonate (MMS) or after maintaining cells at confluence (21). Moreover, only a small increase in *mda-7* mRNA expression occurs in H0-1 human melanoma cells following growth in serum-free medium for 96 hr (21). The difference in regulation of *mda-7* versus the *gadd*, *MyD118* and *gas-1* genes indicates that *mda-7* may represent a new class of growth arresting genes.

In summary, a negative growth regulator, *mda-7*, is described that induces growth suppression in human cancer cells containing both normal and mutated p53 and RB

- 23 -

genes. Characterization of the genomic structure of *mda-7* will be important in determining if this gene normally functions as a tumor suppressor gene and whether alterations are present in this gene in tumor versus normal cells. Identification of the promoter region of *mda-7* will also permit an analysis of the mechanism by which this gene is differentially expressed and inducible by IFN- β plus MEZ in specific cell types. Of potential importance and warranting expanded studies, is the finding that *mda-7* is more growth inhibitory toward cancer and transformed cells than normal cells. In this context, *mda-7* could prove useful as part of a gene-based interventional strategy for cancer therapy, in an analogous manner as the wild-type p53 gene is currently being tested for efficacy in the therapy of specific human malignancies.

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Second Series of Experiments

Melanoma Differentiation Associated Gene-7 (*mda-7*) in a Recombinant Adenovirus Inhibits the Growth of Established Human Tumors in Nude Mice

5

Previous studies document that ectopic expression of *mda-7* in human tumor cells of diverse origins inhibits growth, as documented by a decrease in colony formation in monolayer culture (Jiang et al., PNAS, 93: 9160-9165, 1996). In contrast, *mda-7* does not significantly alter the growth of normal human epithelial or fibroblast cells. These observations support the hypothesis that *mda-7* is a ubiquitous cancer growth suppressor gene.

15 The ability of *mda-7* to selectively inhibit cancer cell growth suggests that this gene might provide therapeutic benefits in the treatment of human cancers. To explore this possibility a replication-defective adenovirus expressing *mda-7* has been generated. The protocols were similar to those used to construct an adenovirus expressing antisense *mda-7*, Ad.*mda-7* AS (Jiang et al., PNAS, 93: 9160-9165, 1996). The recombinant replication-defective Ad.*mda-7* S was produced in two steps. First, the *mda-7* gene was cloned in a sense orientation into a modified Ad expression vector pAd.CMV. This virus contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus (CMV) immediate early promoter, DNA encoding a poly A signal sequence from the beta globin gene, and approximately 3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the CMV immediate early gene promoter, and appropriate RNA processing. The recombinant virus was created in vivo in 293 cells by homologous recombination between *mda-7* containing vector and JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322. JM17 gives rise

to Ad genomes *in vivo* but they are too large to package. This constraint is relieved by recombination with the vector to create a packageable genome, containing the gene of choice. The recombinant virus is replication defective in human cells except 293, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures.

As observed with transfection with *mda-7*, infection of diverse human cancer cell lines, but not normal cell lines, with Ad.*mda-7 S* inhibited growth. These results demonstrate that this virus retains properties observed with the *mda-7* plasmid construct. In many cancer cells, including breast carcinoma (MCF-7 and T47D), glioblastoma (GBM-18 and T98G) and melanoma (H0-1 and C8161), infection with Ad.*mda-7 S* resulted in the induction of programmed cell death (apoptosis). This effect was not elicited in normal cells even after infection with high multiplicities of infection (100 pfu/cell) with Ad.*mda-7 S*. In other cancer cell types, growth suppression (as indicated by a suppression in colony formation in monolayer culture) was apparent without signs of apoptosis, as indicated by nuclear morphology changes, formation of nucleosomal ladders or a positive TUNEL reaction. These results indicate that the Ad.*mda-7 S* virus can selectively inhibit the growth of human cancer cells *in vitro*. Moreover, in specific cancer cell types growth suppression correlates with induction of apoptosis. These observations suggest that inhibition in cancer growth induced by *mda-7* can occur by multiple pathways.

Nude mouse human tumor xenograft models were used to determine if Ad.*mda-7 S* can inhibit the growth of human

cancer cells *in vivo*. Athymic nude mice, obtained from Taconic Labs, were injected subcutaneously with one million human cervical carcinoma (HeLa) cells in PBS mixed with matrigel (final volume 0.4 ml; ratio of matrigel to PBS 1:1). Tumors were allowed to grow until they reached an average volume of 100 to 200 mm³ (10 to 21 days post inoculation). Mice were then randomized and divided into two groups: Group 1: replication-defective Ad lacking the *mda-7* gene; null virus (null); and Group 2: Ad.*mda-7* S. Treatment consisted of intratumoral injections of the null or Ad.*mda-7* S (100 μ l at 4 sites/injection) three times a week for 4 weeks. Tumors were measured twice to three times weekly with a caliper. Tumor volumes were calculated using the formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. After 4 weeks of therapy, animals were followed for an additional week and sacrificed. Final tumor volume divided by initial tumor volume equals tumor volume ratio which is defined as a measure of cancer progression.

Well-established HeLa xenografts, treated with Ad.*mda-7* S, were growth inhibited over the course of the study, whereas tumors treated with the null virus continued to grow progressively (Figures 5 and 6). The *mda-7* inhibitory effect was significant with a p value < 0.05. This study was repeated and similar results were obtained. This data suggest that ectopic expression of *mda-7* may provide therapeutic benefit for the treatment of human cancer. Experiments are now in progress using established human breast cancer tumors, MCF-7 and T47D, in nude mice.

What is claimed is:

1. A method for reversing the cancerous phenotype of a cancer cell which comprises introducing a nucleic acid comprising a melanoma differentiation associated gene (*mda-7*) into the cell under conditions permitting the expression of the gene so as to thereby reverse the cancerous phenotype of the cell.
2. A method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing a nucleic acid comprising a melanoma differentiation associated gene (*mda-7*) into the subject's cancerous cell under conditions permitting expression of the gene in the subject's cell so as to thereby reverse the cancerous phenotype of the cell.
3. The method according to claim 1 or 2, wherein the nucleic acid comprises a vector.
4. The method according to claim 1 or 2, wherein the melanoma differentiation associated gene (*mda-7*) is linked to a regulatory element such that its expression is under the control of the regulatory element.
5. The method according to claim 4, the regulatory element is inducible or constitutive.
6. The method according to claim 4, wherein the regulatory element is a tissue specific regulatory element.
7. The method of claim 1 to 6, wherein the nucleic acid is introduced into the cells by naked DNA technology, adenovirus vector, adeno-associated

- 31 -

virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retroviral vectors, vaccinia virus vector, liposomes, antibody-coated liposomes, mechanical or electrical means.

5

8. The method of claim 1 to 7, wherein the cancer cell is characterized by the presence within it of a defective tumor suppressor gene.

10

9. The method of claim 8, wherein the tumor suppressor gene is a p53, a retinoblastoma (RB) or a p16^{ink4a} gene.

15

10. The method of claim 1 to 7, wherein the cancer cell is characterized by the presence within it of a dominant acting oncogene.

20

11. The method of claim 10, wherein the dominant acting oncogene is Ha-ras, mutant p53 or human papilloma virus gene.

25

12. The method of claim 3, wherein the vector is an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector or vaccinia virus vector.

30

13. The method of claim 12, wherein the adenovirus vector is a replication-defective adenovirus vector expressing *mda-7*, designated Ad.*mda-7* S.

35

14. A method for reversing the cancerous phenotype of a cancer cell which comprises introducing the gene product of a melanoma differentiation associated gene (*mda-7*) into the cancerous cell so as to thereby reverse the cancerous phenotype of the cell.

- 32 -

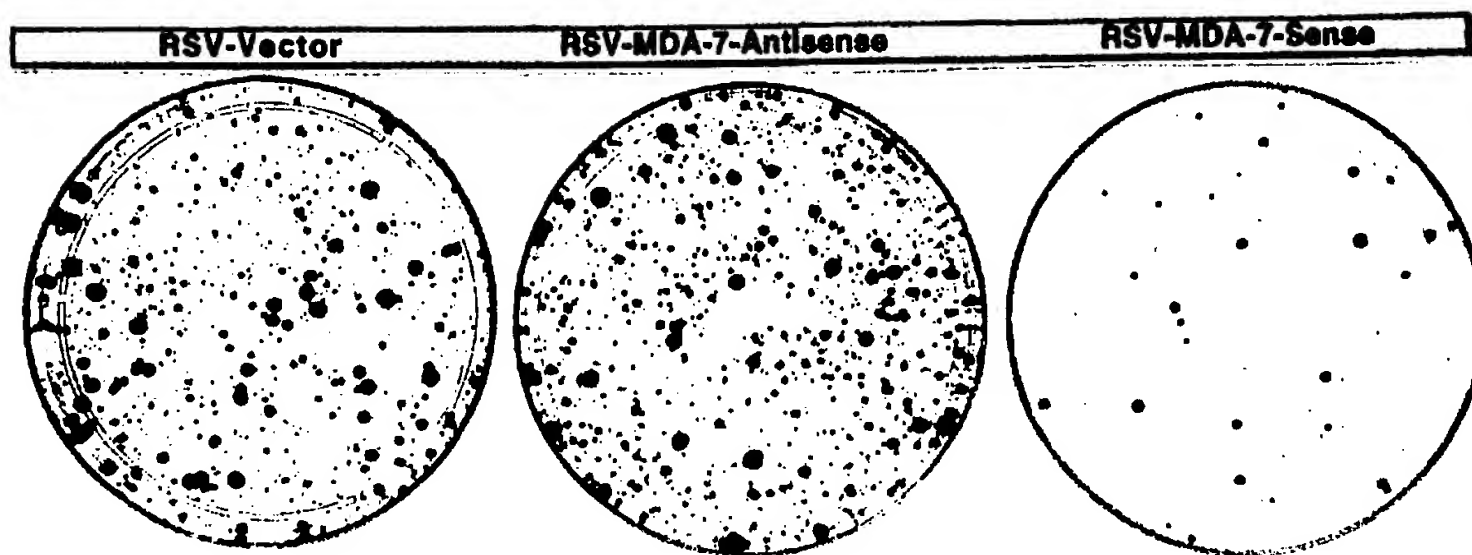
15. A method for reversing the cancerous phenotype of a cancer cell in a subject which comprises introducing the gene product of a melanoma differentiation associated gene (*mda-7*) into the subject's cancerous cell so as to thereby reverse the cancerous phenotype of the cell.
16. The method according to claim 1 to 15, wherein the cancer cell is a breast, cervical, colon, prostate, nasopharyngeal, lung, glioblastoma multiforme, lymphoma, leukemia, connective tissue or nervous system cell.
17. A pharmaceutical composition which comprises an amount of a nucleic acid comprising melanoma differentiation associated gene (*mda-7*) effective to reverse the cancerous phenotype of a cancer cell and a pharmaceutically acceptable carrier.
18. The pharmaceutical composition of claim 17, wherein the nucleic acid comprises a vector.
19. The pharmaceutical composition of claim 18, wherein the vector is an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retrovirus vector or vaccinia virus vector.
20. The pharmaceutical composition of claim 19, wherein the adenovirus vector is a replication-defective adenovirus vector expressing *mda-7*, designated Ad.*mda-7* S.
21. A pharmaceutical composition comprising an amount of the gene product of a melanoma differentiation associated gene (*mda-7*) effective to reverse the cancerous phenotype of a cancer cell and a

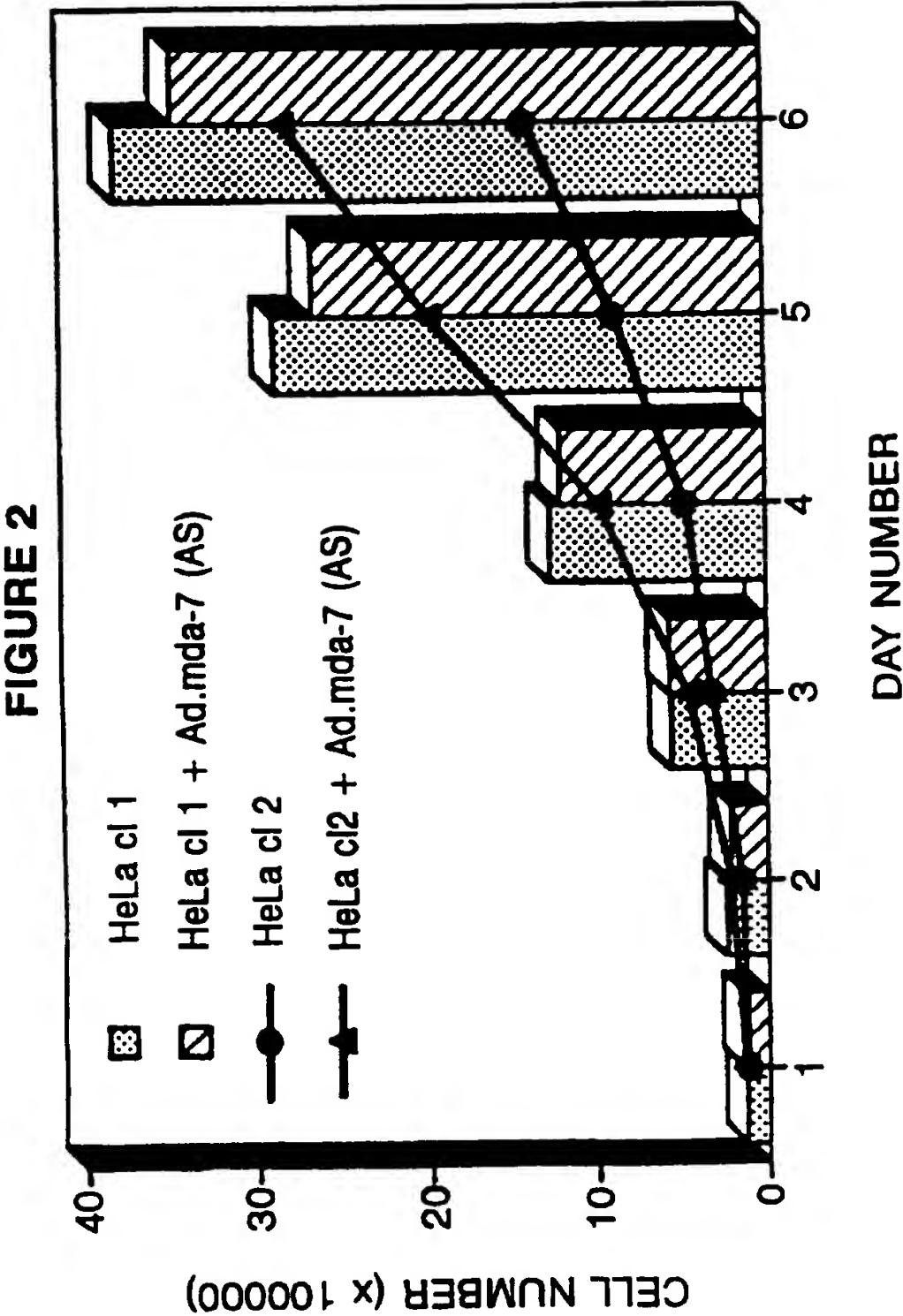
pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim 17 to 21,
wherein the cancer cell is a breast, cervical,
5 colon, prostate, nasopharyngeal, lung, glioblastoma
multiforme, lymphoma, leukemia, connective tissue or
nervous system cell.

1/6

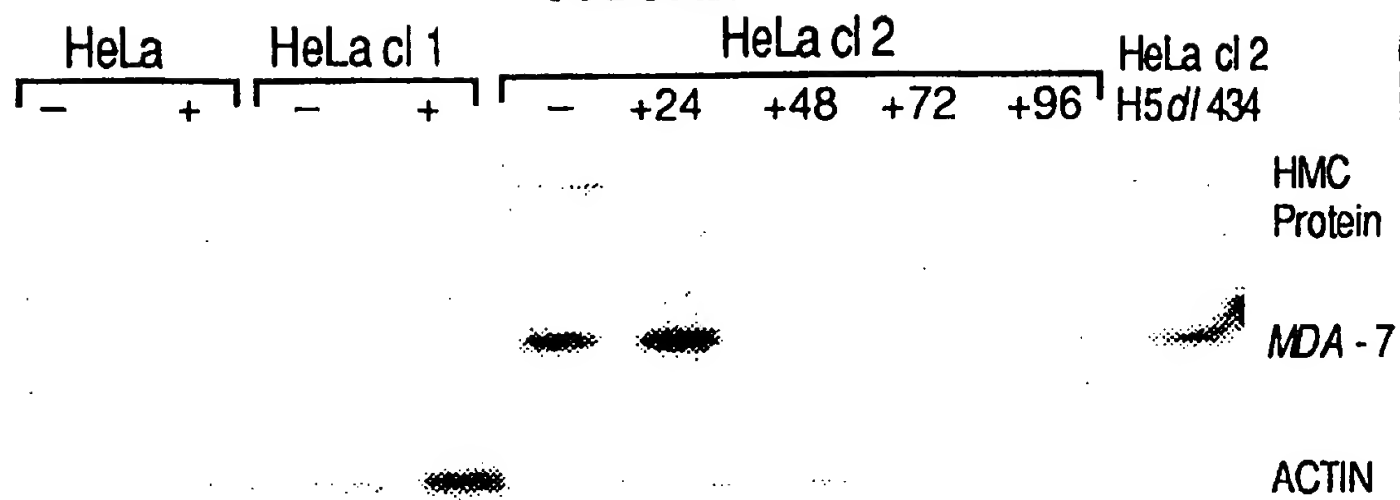
FIGURE 1





3/6

FIGURE 3



4/6

FIGURE 4A

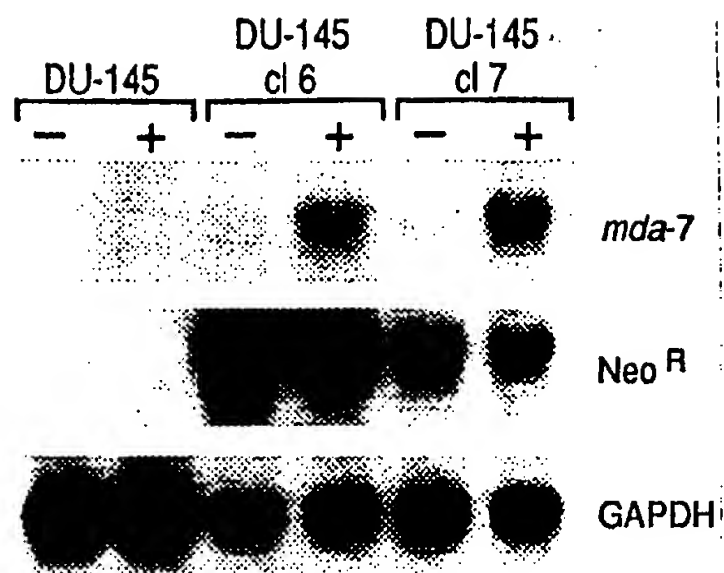
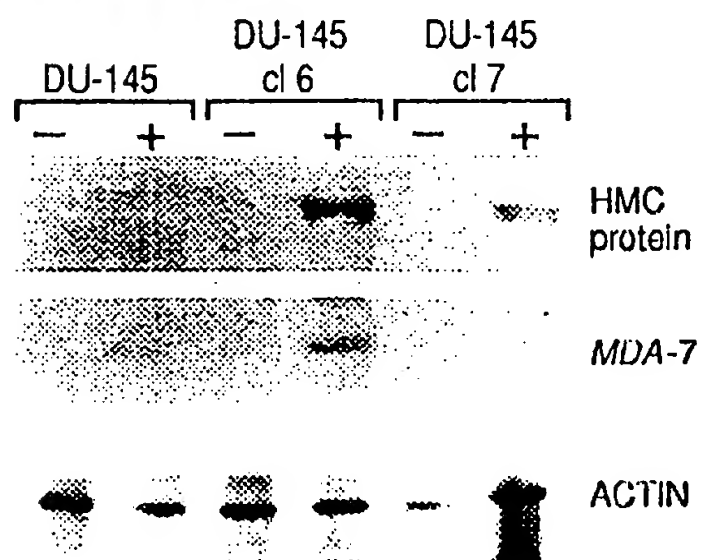


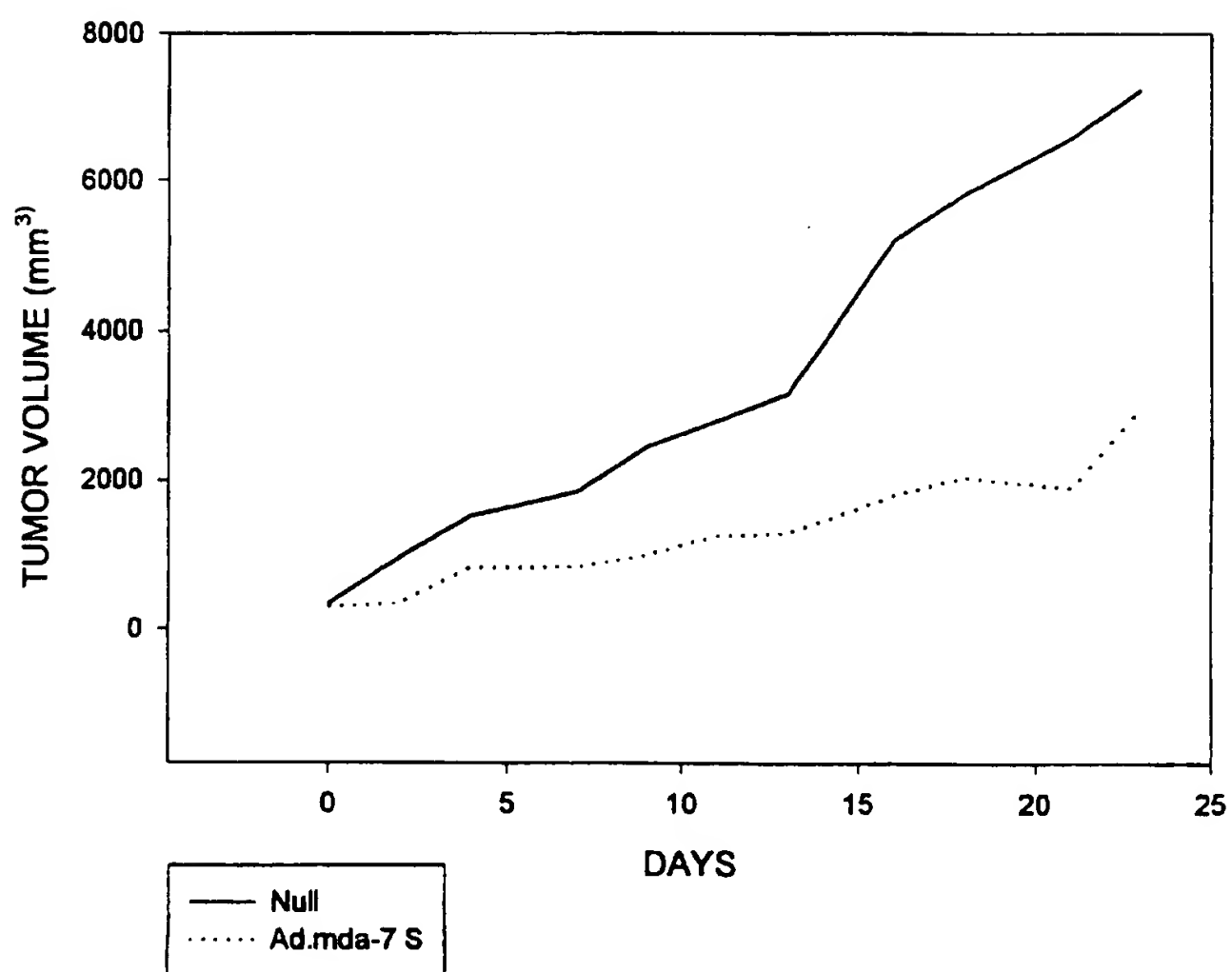
FIGURE 4B



5/6

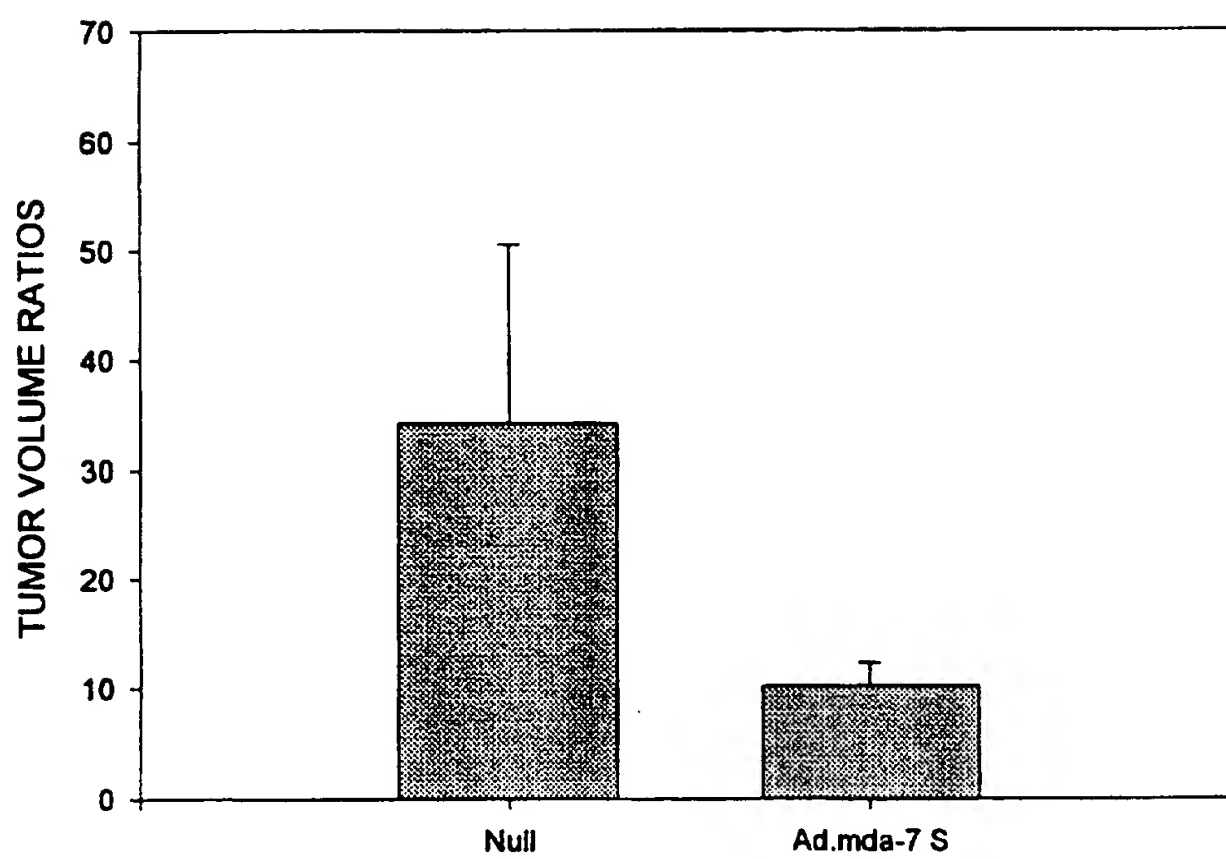
FIGURE 5

Growth Of HeLa Cells Treated With Ad.mda-7 S



6/6

FIGURE 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14548

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00

US CL : 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1; 514/44; 536/23.1, 24.1, 24.3, 24.31, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JIANG et al. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. Proceedings of the National Academy of Sciences, USA. August 1996, Volume 93, Number 17, pages 9160-9165, see especially the abstract.	1-6, 12-14, 16-22
Y,P	US, 5,643,761 A (FISHER et al.) 01 July 1997, see especially column 10, line 47, through column 11, line 35.	1-6, 12-14, 16-22
A	US 5,480,968 A (KRAUS et al.) 02 January 1996, see especially Figure 12 and column 13, lines 48-53.	1-6, 12-14, 16-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* B* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 14 OCTOBER 1997	Date of mailing of the international search report 29 OCT 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14548

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-11 and 15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/14548

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog covering CAS, BIOSIS, MBASE, WPI, BIOTECH ABS, & MEDLINE with search terms: mda, 7, melanoma, differentiation, associated, gene, therapy, antisense